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Structure and activity of the Cas3 HD nuclease MJ0384, an effector enzyme of the CRISPR interference

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1st Editorial Decision 27 July 2011

Thank you for submitting your manuscript for consideration by The EMBO Journal. It has been now been evaluated by three referees and I enclose their reports below.

As you will see the referees express interest in the structural and biochemical characterisation of Cas3 from M. jannaschii. However, some further insight is requested referee #3 with the use of the described mutants to demonstrate the direct role of Cas3 in the cleavage of DNA and RNA substrates and also to identify distinct regions that are responsible for the binding of RNA or DNA and cleavage of these nucleic acids. In addition, a very recent paper (Mulepati and Bailey (2011) JBC) has been brought to my attention describing the structure of a CasHD, it is therefore, very important that these revisions are dealt with in an expedited manner. I would be grateful if you could inform me how long these revisions are likely to take. Given the interest in the study, should you be able to address the concerns we would be happy to consider a revised version of the manuscript for publication in The EMBO Journal.

I should remind you that it is EMBO Journal policy to allow a single round of revision only and that, therefore, acceptance or rejection of the manuscript will depend on the completeness of your responses included in the next, final version of the manuscript. When you submit a revised version to the EMBO Journal, please make sure you upload a letter of response to the referees' comments. Please note that when preparing your letter of response to the referees' comments that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process initiative, please visit our website: http://www.nature.com/emboj/about/process.html

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

Yours sincerely,

Editor
The EMBO Journal

REFEREE REPORTS

Referee #1

Beloglazova and coworkers describe the structure and activity of the HD-type nuclease that plays an important role in CRISPR/Cas interference. It is demonstrated that the M.jannaschii protein (MJ0384; Cas3') has both endo- and exo-nuclease 3'-to-5' activity on single stranded DNA and RNA (as part of different complex-types). R-loop structures, the anticipated physiological target, are demonstrated to be degraded due to the enzyme's subsequent endo- and exo-nucleolytic cleavage of ssDNA. The nuclease activity is Mg-dependent/EDTA-sensitive, and is stimulated by the Cas3' helicase in a Mg/ATP-dependent manner, a domain that is fused to the HD domain in the majority of Type-I CRISPR/Cas systems. The crystal structure of the monomeric HD domain reveals a typical HD fold, albeit with a distinct catalytic metal coordination and a relatively well exposed active site. This is a solid molecular study, well described, and a step forward in our understanding of the mechanism of this fascinating defense system.

Comments

1. The authors should indicate what peptide was removed upon Thermolysin treatment, both in the text on p12 (line 10) and in the legend of Fig.S1; now this information is presented at the end of the Results section (p.15 Leu215-Ile 244); as this peptide carries the Asp219 active site residue, how does this proteolysis affect the conclusion on the unusual "large open active site" ; authors should comment on this, and possibly speculate on the apparent flexible nature of this peptide (clamp/lid).

2. Features of nuclease and helicase (substrates, metal/ATP dependence) should be compared more extensively to recently published data by Sinkunas et al. (2011; Ref 20)

3. To improve the readability, the description of the analysis of site-directed mutants (p. 13) should be extended with the details on the actual substrate (probably linear ssDNA; what size/sequence?), preferably in the beginning of the paragraph.

Minor comments
4. P6, line 17: against the both > against both
5. P10, line 11: co-localized or sometimes fused > co-localized, and in many cases even fused
6. P11, line 19: the phosphodiesterase > the human phosphodiesterase
7. P16, line 15: jannashii > jannaschii

Referee #2

A thorough biochemical and structural investigation of Cas3 HD nuclease (along with some biochemical data on its partner helicase protein) is reported. The experiments are well controlled, clearly described, and strong evidence was provided for the main conclusions drawn. While the work is not the first to describe Cas3 and partner protein as a nuclease/helicase system that cleaves single-stranded nucleic acids non-specifically (e.g. Siksyns' group reported this earlier on the Cas3 protein from S. thermophilus), the work significantly extends our understanding of the action of Cas3. Given Cas3's proposed role as an effector protein mediating defense of type 1 CRISPR/Cas immune systems combined with the general excitement for the area of research, this paper will have both broad appeal as well as make an important contribution to the field of CRISPR research.

Minor comments and suggestions:

1. Title: change "killer" to "effector".

2. Abstract (line 10): Change "proposed" to "suggested" or "indicated".

3. Introduction (top of page 4, paragraphs one and two): include the Cas RAMP module (Cmr) set of Cas proteins as a module of CRISPR/Cas immunity and indicate (with appropriate citations) that this module targets RNA and lacks Cas3. Also, on page 6, first sentence of second paragraph, "unknown" should be changed to "not fully characterized" (or similar phrase that avoids the overstatement).

4. The recently published work on E. coli Cas3 by Edward Bolt's group (Biochemical Journal, entitled "Helicase dissociation and annealing of RNA-DNA hybrids by E. coli Cas 3 protein" should be cited in introduction as well as acknowledged elsewhere (e.g. top of page 16 of discussion) where appropriate.

5. Page 11, second paragraph, line 6. The data is not shown to support the gel filtration data presented (show the data or at least indicate "data not shown" to avoid confusion by the reader).

6. Page 12, last paragraph, line 5. Change "artefact" to "artifact".

7. Author contributions, line 4 there is an "XX" that needs to be replaced by the name(s) of researchers.

8. Page 20, last line of first paragraph. Change "SIBR" to SYBR".

Referee #3

Cas3 proteins are thought to participate in CRISPR-based microbial immunity against phage and plasmids by cleaving invasive DNA. In this study, Yakunin and colleagues present evidence that the Cas3 HD domain protein from M. jannaschii cleaves endonucleolytically and exonucleolytically (3′ to 5′) both single-stranded DNAs and RNAs, as well as 3′-flaps, splayed arms, and R-loop structures. The degradation of branched DNA substrates by Cas3 HD is stimulated by a Cas3 helicase protein and ATP. The crystal structure of Cas3 HD shows that the active site contains two bound metal cations; site-directed mutagenesis allowed the authors to propose a possible catalytic mechanism. The results suggest that Cas3 HD nucleases could function together with Cas3-type helicases to catalyze complete degradation of foreign DNA molecules through a combination of endo- and exonucleolytic activities.

Overall this is an interesting study that sheds new light on possible biochemical functions of Cas3 enzymes, which are emerging as important players in the CRISPR-mediated destruction of invading nucleic acids. However, there are several important issues that need to be addressed in order to clarify the interpretation of the data:

1. In the activity assays shown in Figs. 1 and 2 (and related supplemental figures), purified Cas3 proteins apparently cleave both DNA and RNA substrates, have both magnesium-dependent (ssDNA) and magnesium-independent (ssRNA) activities, and can cleave both endo- and exonucleolytically. Additional controls are required to rule out that some of these observed activities result from contaminating nucleases present in the reactions. Although point mutants are tested later (Fig. 4), one or more should be included in these panels, and for both DNA and RNA substrates. Also, the authors should comment on the products of the reactions, which appear non-uniform over the timecourse of the reactions. For example, in Fig. 2a a ~25 nt product appears after 15 minutes of incubation and a 12-nt product is predominant after an additional 5 minutes of incubation. Are these discrete products due to sequence or structural features of the substrates, or do they reflect a "footprint" of the protein on the substrate molecule? Are these products relevant to the behavior of Cas3 within the CRISPR-based targeting complexes responsible for target recognition? And does Cas3 helicase change the product profile?

2. Related to the above point, how do the authors envision that Cas3 activities are regulated? If this nuclease is really as promiscuous as these in vitro data would suggest, I don't understand how its

actions are controlled in vivo. Are there other examples of nucleases with such broad substrate specificity and catalytic activity?

3. Fig. 2b: degradation rates appear to be relatively slow; were kinetic rate constants measured for these reactions? Are the two types of products (long vs. short) produced at different rates? Also, why was a 39-nt. complementary RNA used in these experiments - is this the size of the crRNA in M. jannaschii?

4. Fig. 4: were the 4 mutant proteins containing disrupted metal ion binding sites tested for cleavage of RNA substrates? A prediction based on the author's interpretation of the Fig. 1 and 2 data is that RNA substrates should continue to be cleaved by these mutants, whereas mutations affecting other parts of the protein might affect DNA only, or both DNA and RNA cleavage. The conclusions about Cas3HD specificity and activity would be much more convincing if the authors could use their extensive mutant collection to identify distinct DNA and RNA binding sites and/or distinct active site residues that contribute to DNA versus RNA cleavage.

1st Revision - authors' response 15 August 2011

Referee #1

Question 1 (Q1). *The authors should indicate what peptide was removed upon Thermolysin treatment, both in the text on p12 (line 10) and in the legend of Fig.S1; now this information is presented at the end of the Results section (p.15 Leu215-Ile 244); as this peptide carries the Asp219 active site residue, how does this proteolysis affect the conclusion on the unusual "large open active site" ; authors should comment on this, and possibly speculate on the apparent flexible nature of this peptide (clamp/lid).*
Authors' Response-1 (AR1). We added this information in the Methods section (p. 23), as well as in the text (p. 14) and corrected it on p. 17 in Results (to Met217-Ile244). We also added a comment about the potential flexible C-terminal strand and its possible function as a molecular clamp (p. 17).

Q2. *Features of nuclease and helicase (substrates, metal/ATP dependence) should be compared more extensively to recently published data by Sinkunas et al. (2011; Ref 20).*
AR2. Additional comparative comments about the *S. thermophilus* Cas3 protein have been added on p. 10 (2nd paragraph) and p.15 (2nd paragraph).

Q3. *To improve the readability, the description of the analysis of site-directed mutants (p. 13) should be extended with the details on the actual substrate (probably linear ssDNA; what size/sequence?), preferably in the beginning of the paragraph.*
AR. As suggested, more details have been added in this paragraph about the substrates used: linear ssDNA (40 nt) and ssRNA (39 nt) (now on p. 15, 2nd paragraph).

Q4. *P6, line 17: against the both > against both*
AR. Corrected as suggested.

Q5. *P10, line 11: co-localized or sometimes fused > co-localized, and in many cases even fused*
AR. Corrected as suggested.

Q6. *P11, line 19: the phosphodiesterase > the human phosphodiesterase*
AR. Corrected as suggested

Q7. *P16, line 15: jannashii > jannaschii*
AR. Corrected.

Referee #2

Q1. *Title: change "killer" to "effector".*

AR. Initially, we thought to use "effector" in the title, but this term is widely used for a large group of pathogenic microbial proteins which are exported into eukaryotic cells and use various mechanisms to suppress innate immunity. Therefore, its use for a CRISPR-associated protein which is part of a host immunity pathway will be confusing. We agree that the term "killer" is a bit awkward, but we have no a better substitute.

Q2. *Abstract (line 10): Change "proposed" to "suggested" or "indicated".*
AR. Changed to "suggested".

Q3. *Introduction (top of page 4, paragraphs one and two): include the Cas RAMP module (Cmr) set of Cas proteins as a module of CRISPR/Cas immunity and indicate (with appropriate citations) that this module targets RNA and lacks Cas3.*
AR. Indicated as suggested (p. 4, 1st paragraph, last sentence).

Q4. On page 6, first sentence of second paragraph, "unknown" should be changed to "not fully characterized".
AR. This is on page 5 – corrected as suggested.

Q5. *The recently published work on E. coli Cas3 by Edward Bolt's group (Biochemical Journal, entitled "Helicase dissociation and annealing of RNA-DNA hybrids by E. coli Cas 3 protein" should be cited in introduction as well as acknowledged elsewhere (e.g. top of page 16 of discussion) where appropriate.*
AR. This work is now cited in Introduction (p. 5, 2nd paragraph), Results (p. 11, 1st paragraph), and Discussion (p. 19, 2nd paragraph).

Q6. *Page 11, second paragraph, line 6. The data is not shown to support the gel filtration data presented (show the data or at least indicate "data not shown" to avoid confusion by the reader).*
AR. As suggested, we added "data not shown" to this sentence.

Q7. *Page 12, last paragraph, line 5. Change "artefact" to "artifact".*
AR. This sentence is changed, and this word is removed.

Q8. *Author contributions, line 4 there is an "XX" that needs to be replaced by the name(s) of researchers.*
AR. Corrected.

Q9. *Page 20, last line of first paragraph. Change "SIBR" to SYBR".*
AR. Corrected as suggested.

Referee #3

Q1. *In the activity assays shown in Figs. 1 and 2 (and related supplemental figures), purified Cas3 proteins apparently cleave both DNA and RNA substrates, have both magnesium-dependent (ssDNA) and magnesium-independent (ssRNA) activities, and can cleave both endo- and exonucleolytically. Additional controls are required to rule out that some of these observed activities result from contaminating nucleases present in the reactions. Although point mutants are tested later (Fig. 4), one or more should be included in these panels, and for both DNA and RNA substrates. Also, the authors should comment on the products of the reactions, which appear non-uniform over the time course of the reactions. For example, in Fig. 2a a ~25 nt product appears after 15 minutes of incubation and a 12-nt product is predominant after an additional 5 minutes of incubation. Are these discrete products due to sequence or structural features of the substrates, or do they reflect a "footprint" of the protein on the substrate molecule? Are these products relevant to the behavior of Cas3 within the CRISPR-based targeting complexes responsible for target recognition? And does Cas3 helicase change the product profile?*
AR. As requested by this referee, the results with two MJ0384 mutant proteins (D67A and H123A) have been added to Fig. 1B and Suppl. Fig. S2D. Additionally, RNase activity of the 10 purified MJ0384 mutant proteins was analyzed and the results are shown in Suppl. Fig. S3H. Both RNase and DNase activity profiles look similar, and this is indicated in the text (p.16, 1nst paragraph, last sentence). We also added a comment (p. 7, 2nd paragraph) on the accumulation of intermediate

reaction products of ssDNA cleavage (shown in Fig. 1B-E). Analysis of the product profiles and sequences of the DNA substrates used in these experiments (34-91 nt) suggests that the observed cleavage pattern is likely to be a combined effect of the intermittent secondary structures formed during the assay and a "footprint" of the protein on the substrate molecule. For some long ssDNA substrates, the addition of a Cas3 helicase can potentially affect the MJ0384 product profile, and the detailed characterization of the activity of Cas3 helicase MJ0383 and its interactions with the HD nuclease MJ0384 in DNA cleavage will be addressed in our future work.

Q2. *Related to the above point, how do the authors envision that Cas3 activities are regulated? Are there other examples of nucleases with such broad substrate specificity and catalytic activity?*
AR. We agree that the ability of Cas3 HD nucleases to cleave both ssDNAs and ssRNAs non-specifically is potentially dangerous for host cells and needs to be tightly regulated both at the level of gene expression and enzyme activity (e.g. through complexes with other proteins or metal availability). We added this comment in Discussion (p. 18, 1st paragraph). Many nucleases have been shown to cleave both RNA and DNA and are discussed in several reviews (e.g. PMID:21225639). For example, apurinic/apyrimidinic endonuclease 1 (APE1, the major mammalian enzyme of DNA base excision repair) cleaves ssRNAs and abasic ssDNAs and ssRNAs in vitro (PMID:21762700).

Q3. *Fig. 2b: degradation rates appear to be relatively slow; were kinetic rate constants measured for these reactions? Are the two types of products (long vs. short) produced at different rates? Why was a 39-nt. complementary RNA used in these experiments - is this the size of the crRNA in M. jannaschii?*
AR. Yes, the reaction rates for the formation of short products are lower than those for long products, because the former are initiated by an endonuclease cleavage of ssDNA which is slower than the exonuclease activity.
Yes, 39 nucleotides is the median size of the *M. jannaschii* CRISPR spacer (36-41 nt).

Q4. *Fig. 4: were the 4 mutant proteins containing disrupted metal ion binding sites tested for cleavage of RNA substrates? A prediction based on the author's interpretation of the Fig. 1 and 2 data is that RNA substrates should continue to be cleaved by these mutants, whereas mutations affecting other parts of the protein might affect DNA only, or both DNA and RNA cleavage. The conclusions about Cas3HD specificity and activity would be much more convincing if the authors could use their extensive mutant collection to identify distinct DNA and RNA binding sites and/or distinct active site residues that contribute to DNA versus RNA cleavage.*
AR. Our additional experiments demonstrated that the four metal-coordinating residues of MJ0384 (Asp67, His91, His123, and His124) are important for ssRNA cleavage. In addition, six other mutant proteins showed similar activities for both ssDNA and ssRNA cleavage, suggesting that all activities are associated with the same active site. This is now indicated in the text (p. 16, 1st paragraph) and shown in Suppl. Fig. S3H. The detailed characterization of MJ0384 RNase activity and its relationships with DNase activity will be addressed in our future work.

2nd Editorial Decision 02 September 2011

Thank you for submitting your revised manuscript for consideration by the EMBO Journal. It has now been seen by two of the referees whose comments are enclosed. As you will see, both recommend publication, I suggest that you make the text changes and email us a new version of the Word document by email as soon as possible.

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

Yours sincerely,

Editor
The EMBO Journal

REFEREE REPORTS

Referee #2

The authors have satisfactorily addressed all main concerns of the reviewers and the manuscript is ready for publication.

Two minor comments:
1. Page 5, second sentence from bottom (ions to ion)
2. The argument for retaining the slang term "killer" in the title vs another term (e.g. "effector" is weak ("effector" is a general term used to describe a component that mediates a specific effect - it is used commonly in several fields not just in reference to the proteins mentioned.) I remain unconvinced that such vernacular should be promoted in the title of this paper or that readers will be confused by the use of the phrase "effector" (or comparable adjective).

Referee #3

The authors have adequately addressed the concerns of the reviewers in the revised manuscript.

